GENETIC MARKER FOR CORONARY ARTERY DISEASE

FIELD OF THE INVENTION

The present invention describes a coronary artery susceptibility gene and its use in the diagnosis and therapy of CAD.

BACKGROUND

Coronary artery disease (CAD) is the most common type of heart disease. It is a consequence of atherosclerosis, which is a progressive inflammatory tissue response resulting from deposition of modified lipoproteins in the arterial intima of the vascular wall. This process can lead to development of complex atherosclerotic lesions or plaques at pre-existing focal arterial intima cushions rich in extracellular matrix (Glass et al, Cell 104, 503-516, 2001). Overtime these lesions narrow the coronary arteries, restricting blood flow to the heart and can cause angina. Complete blockage can lead to myocardial infarction.

The known risk factors for CAD include type II diabetes, insulin resistance, obesity (Rao *et al*, Am. Heart J. 142, 1102-1107, 2001), hyperlipidemia, high blood pressure, cigarette smoking and physical inactivity. CAD is also known to have a significant genetic component.

There are no drugs currently available to prevent CAD. Efforts have been focussed at measures to slow down CAD, and different drugs and surgical techniques are available to repair clogged coronary arteries. However, there is a need for a better understanding of the pathophysiology of CAD and the development of specific and effective drugs.

To develop more effective drugs, it is critical that the susceptibility genes for CAD are identified. As mentioned above, the pathogenesis of CAD has been shown by twin and other studies to include a significant genetic component, and the effects of genes on susceptibility to CAD are likely to be particularly strong at younger ages (Marenberg *et al*, New England J Med 330, 1041-1046, 1994). However, CAD is unlikely to be inherited as a simple Mendelian trait, but is instead a complex multifactorial disorder in which the phenotype is heavily influenced by environmental as well as genetic factors. Although a number of loci have been identified for risk factors related to CAD, only a limited number of genome scanning studies have been published showing linkage to CAD. For example, CAD has been

linked to chromosome 2 and X (Pajukanta et al, Am. J. Hum. Genet. 67, 1481-1493, 2000), chromosome 16 (Francke *et al.*, Human Molec. Genet. 10, 2751-2765, 2001) and chromosome 14 (Broeckel *et al.*, Nature Genetics 30, 210, 2002).

SUMMARY OF THE INVENTION

The present invention describes the identification of a CAD susceptibility gene, the oxytocin receptor (OXTR). The identification of OXTR as a CAD susceptibility gene enables the development of novel therapies for CAD by screening for compounds and other entities, such as antibodies, which modulate the activity of OXTR. Knowledge of the OXTR gene sequence also enables the development of novel antigene methods to modulate the expression of the associated gene and may also enable the development of novel gene therapy techniques to treat CAD. The discovery of the linkage between OXTR and CAD may also assist in developing novel methods for diagnosing CAD via (i) measuring the levels of the translated mRNA of OXTR present in affected tissue and (ii) measuring the levels of the OXTR protein in affected tissue. It is possible that the diagnosis of CAD, or the susceptibility of an individual to CAD, by these methods may be achieved in patients who do not yet display the classical symptoms of the disease. Such determination of susceptibility to CAD, or the early detection of disease development, will lead to earlier clinical intervention than is currently possible and will lead to more effective treatment of the disease.

Specifically, the invention includes a method of identifying a test compound that modulates the expression of an OXTR gene, includes contacting a cell capable of expressing an OXTR gene with a test compound; and determining the level of expression of the OXTR gene in the presence of the test compound, wherein a decrease or an increase in OXTR gene expression, as compared to the level of expression of OXTR in the absence of the compound, is indicative that the test compound modulates OXTR gene expression.

Also within the invention is a method of identifying a test compound that modulates the activity of a protein encoded by the OXTR gene, including contacting the protein with a test compound and determining the level of activity of the OXTR protein in the presence of the compound, wherein a decrease or an increase in the protein activity, as compared to the level

of activity of the OXTR protein in the absence of the compound, is indicative that the test compound modulates OXTR protein activity.

The invention further includes a method for determining if an OXTR gene has an altered level of gene expression in a CAD cell. The method includes comparing the level of OXTR gene expression in a cell from a patient having CAD with a control cell (a cell from a patient not having CAD), and determining the level of expression of the OXTR gene in both cells, wherein a decrease or an increase in expression of the OXTR gene, as compared to the level of expression of the OXTR gene in the control cell, indicates that the OXTR has altered gene expression.

Also within the invention is a method for determining the level of an OXTR protein in a CAD patient compared to a control including comparing the protein level of OXTR in a cell from a patient having CAD with a control cell (a cell from a patient not having CAD), and determining the level of the OXTR protein in both cells.

In another aspect, the invention includes a method of identifying a binding partner of the OXTR protein including contacting an OXTR protein with a test target protein, and determining if the test target protein can interact with the OXTR protein, wherein interaction of the test target protein with OXTR indicates that the test target protein is an OXTR binding partner. In one embodiment, the method can further include contacting a gene encoding the test target protein with a test compound; and determining the level of expression of the test target gene in the presence of the test compound, wherein a decrease or an increase in test target gene expression, as compared to the level of expression of the test target gene in the absence of the compound, is indicative that the test compound modulates expression of the test target gene and is useful in the treatment of CAD. In another embodiment, the method further includes contacting the test target protein with a test compound; and determining the level of activity of the test target protein in the presence of the test compound, wherein a decrease or an increase in test target protein activity, as compared to the level of activity of the test target protein in the absence of the compound, is indicative that the test compound modulates test target protein activity and is useful in the treatment of CAD.

The invention further features a method of treating a subject having CAD comprising administering an effective amount of the compound identified above. The invention further includes a pharmaceutical composition comprising the compound identified as above.

In another aspect, the invention includes a method of identifying other components of the CAD biochemical pathway of which OXTR is a component.

The invention further includes methods of diagnosing CAD or a susceptibility thereto in a subject. The method includes determining the level of an OXTR protein in a sample from a subject; and comparing the level of the protein in the sample with a control, wherein a decrease or an increase in the level of the protein in the sample compared to the control indicates that the subject has CAD, or a susceptibility thereto. The invention also extends to products useful for carrying out the assay, such as DNA probes (labelled or unlabelled), kits and the like.

The present invention also includes gene-based therapies directed at the OXTR gene. Therapies may be in the form of polynucleotides comprising all or a portion of the OXTR gene, placed in appropriate vectors or delivered to target cells in direct ways which would modify the function of the OXTR protein.

As used herein, a "CAD susceptibility gene" refers to a gene that has a predisposing influence on the development of CAD in a subject.

As used herein, "coronary artery disease" (CAD) refers to disorders and conditions related to the deposition of atheroma in the large- and medium-sized arteries serving the heart. Coronary artery disease means clinical syndromes (including, but not limited to, angina, myocardial infarction, unstable angina, and sudden ischemic death) which are based on the pathology of coronary artery atheroma.

As used herein "OXTR protein" refers to the OXTR protein, peptide fragments thereof, mutants, variants, truncated forms of OXTR, and fusion proteins of OXTR.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 is a line graph showing the KAC LOD-score of chromosome 3.

DETAILED DESCRIPTION OF THE INVENTION

The present invention describes a CAD susceptibility gene, oxytocin receptor (OXTR). OXTR is a seven transmembrane G-protein coupled receptor that is encoded by a single gene in the human genome. Oxytocin is well recognised for its role in lactation, parturition, central regulation of sex, maternal behavior and feeding (Gimple and Fahrenholtz Physiol Rev 81:629-83,2001). However, there is substantial data indicating a role of oxytocin and OXTR in regulation of blood volume and natriuresis. The OXTR is expressed throughout the cardiovascular system and has been suggested to involved in the regulation of vascular tone, regrowth and remodeling (Jankowski *et al.*, Proc Natl Acad Sci 23;97:6207-11, 2000). Oxytocin is believed to regulate cardiac rate and force of contraction through the OXTR (Shojo and Kanedo, Mol Genet Metab 71:552-8, 2000; Mukaddam-Daher *et al.*, Hypertension 38:292-6, 2001). Furthermore, OXTR is expressed in human endothelial cells from aorta and several other vessels (Thibonnier *et al.*, Endocrinology, 140:1301-09, 1999). OXTR is also expressed in adipocytes, pancreas, adrenal gland and in many brain areas (Gimple and Fahrenholtz, Physiol Rev, 81:629-83, 2001).

The present finding shows that OXTR, which is mapped to chromosome region 3p, shows linkage with DNA markers in CAD affected individuals. The OXTR gene and amino acid sequences are known for many different species, e.g., the human OXTR nucleic and amino acid sequences are available on genbank (http://www.ncbi.nlm.nih.gov/), under accession number NM 000916. Knowledge of the OXTR sequence enables the development of novel antigene methods to modulate the expression of OXTR and also enables the development of novel gene therapy techniques to treat CAD.

The sequence of human OXTR protein, as disclosed in NM 000916, is depicted in SEQ ID NO: 1 and the cDNA sequence in SEQ ID NO: 2.

Functional Assay

To further evaluate the role of OXTR in CAD, various functional assays can be performed. For example techniques such as Northern analysis, *in situ* hybridization or expression profiling on cDNA microarrays can be used to further verify the association of OXTR with CAD. In one example, a reporter-based assay may be devised to detect whether the OXTR gene has a different transcription level and/or message stability compared to the same gene in a person not susceptibile to CAD. Individuals who carry the OXTR gene may exhibit differences in their ability to regulate protein biosynthesis under different physiological conditions and may display altered physiological abilities.

In another example, the level of OXTR gene expression can be assayed by detecting and measuring OXTR transcription. For example, RNA from a cell type or tissue known, or suspected of having CAD, can be isolated and tested utilizing hybridization or PCR techniques such as those described above. The isolated cells can be obtained from cell culture or from a patient. The analysis of cells taken from culture may be a necessary step in the assessment of cells to be used as part of a cell-based gene therapy technique or, alternatively, to test the effect of compounds on the expression of OXTR. Such analyses can reveal both quantitative and qualitative aspects of the expression pattern of the OXTR gene, including activation or inactivation of OXTR gene expression.

In one embodiment of such a detection scheme, cDNAs are synthesized from the RNAs of interest (e.g., by reverse transcription of the RNA molecule into cDNA). A sequence within the cDNA is then used as the template for a nucleic acid amplification reaction, such as a PCR amplification reaction, or the like. For detection of the amplified product, the nucleic acid amplification may be performed using radioactively or non-radioactively labeled nucleotides. Alternatively, enough amplified product can be made such that the product can be visualized by standard ethidium bromide staining or by utilizing any other suitable nucleic acid staining method.

Additionally, it is possible to perform OXTR gene expression assays *in situ*, i.e., directly upon tissue sections (fixed and/or frozen) of patient tissue obtained from biopsies or resections, such that no nucleic acid purification is necessary (See, e.g., Nuovo, G. J., PCR In Situ

Hybridization: Protocols And Applications, Raven Press, NY, 1992).

Alternatively, if a sufficient quantity of the appropriate cells can be obtained, standard Northern analysis can be performed to determine the level of mRNA expression of the OXTR gene.

Also within the invention is the determination of whether OXTR gene has a gene mutation compared to the wild-type gene. Alternative methods for the detection of OXTR gene mutations in patient samples or other appropriate cell sources, may involve their amplification, for example, by PCR (the experimental embodiment set forth in U.S. Pat. No. 4,683,202), followed by the detection of the amplified molecules using techniques well known to those of skill in the art. The resulting amplified sequences can be compared to those which would be expected if the nucleic acid being amplified contained only normal copies of the OXTR gene in order to determine whether an OXTR gene mutation exists.

Additionally, well-known genotyping techniques can be performed to identify individuals carrying OXTR gene mutations. Such techniques include, for example, TaqMan 5′ nuclease assay, allele specific PCR, primer extension, mass spectrometry and Pyrosequencing (reviewed in Kwok, Pharmacogenomics 1: 95-100, 2000)

Pathway mapping

Also within the invention is the identification and elucidation of the CAD biochemical\signal transduction pathway of which OXTR is a component. In particular, the invention includes identifying the other components of the OXTR CAD biochemical pathway. In this way it is possible to identify the specific critical signaling pathway which links the disease stimulus to the cell's response and enables the identification of new potential targets for therapy intervention.

As used herein, an OXTR "target molecule" is a molecule in the CAD biochemical pathway with which OXTR binds or interacts, directly or indirectly, with, or is a molecule that regulates the expression of the CAD gene or translation of the protein. For example, the target molecule can be a protein which directly interacts with OXTR, or can be a protein

which does not itself directly interact with the OXTR, but which is a component of the OXTR CAD biochemical pathway. In one example, the target molecule can be a cell membrane or a cytoplasmic molecule. In another example, the target molecule can be an intercellular protein or a protein which facilitates the association of downstream or upstream signaling molecules with the OXTR protein.

According to a further aspect of the invention there is provided the use of the OXTR protein in research to identify further gene targets implicated in CAD. Methods for identifying proteins which interact with OXTR are known in the art, e.g., the two-hybrid assay (see, e.g., U.S. Pat. No. 5,283,317; Zervos *et al.* Cell 72:223-232, 1993) or using cell culture techniques to identify binding partners.

The two-hybrid system is based on the modular nature of most transcription factors, which consist of separable DNA-binding and activation domains. Briefly, the assay utilizes two different DNA constructs. In one construct, the gene that codes for an OXTR protein is fused to a gene encoding the DNA binding domain of a known transcription factor (e.g., GAL-4). In the other construct, a DNA sequence, from a library of DNA sequences, that encodes an unidentified protein ("prey" or "sample") is fused to a gene that codes for the activation domain of the known transcription factor. If the "bait" and the "prey" proteins are able to interact, *in vivo*, forming an OXTR-dependent complex, the DNA-binding and activation domains of the transcription factor are brought into close proximity. This proximity allows transcription of a reporter gene (e.g., LacZ) which is operably linked to a transcriptional regulatory site responsive to the transcription factor. Expression of the reporter gene can be detected and cell colonies containing the functional transcription factor can be isolated and used to obtain the cloned gene which encodes the "target" protein which interacts with the OXTR protein.

Alternatively, binding partners for OXTR can be identified using cell culture techniques or using cells obtained directly from a CAD patient. The method includes isolating the OXTR protein from the cell and determining the identity of its target molecule. Initial screening can be accomplished by Western blot analysis to analyse immunochemically, e.g., using

antibodies against the OXTR, the size of OXTR –target molecule complex. Further analysis of the complex will reveal the identity of the target molecule.

The gene and protein encoded by the target molecule is also a potential target for therapeutic intervention in CAD disease, for instance in the development of antisense nucleic acid targeted to the mRNA; or more widely in the identification or development of chemical or hormonal therapeutic agents. The person skilled in the art is also capable of devising screening assays to identify compounds (chemical or biological) that modulate (activate or inhibit) the identified gene, which compounds may prove useful as therapeutic agents in treating or preventing CAD.

OXTR Proteins and Polypeptides

OXTR proteins, peptide fragments, mutated, and truncated forms of the OXTR, and/or OXTR fusion proteins, can be prepared for a variety of uses, including but not limited to the generation of antibodies, as reagents in diagnostic assays, in the identification of other cellular gene products involved in the pathogenesis of CAD, as reagents in assays for screening for compounds that can be used in the treatment of CAD, and as pharmaceutical reagents useful in the treatment of CAD.

A variety of host-expression vector systems may be utilized to express the OXTR nucleotide sequences of the invention. The expression systems that may be used for purposes of the invention include but are not limited to microorganisms such as bacteria (e.g., *E. coli, B. subtilis*) transformed with recombinant bacteriophage DNA, plasmid DNA or cosmid DNA expression vectors containing OXTR nucleotide sequences or mammalian cell systems (e.g., COS, CHO, BHK, 293, 3T3) harbouring recombinant expression constructs containing promoters derived from the genome of mammalian cells (e.g., metallothionein promoter) or from mammalian viruses (e.g., the adenovirus late promoter; the vaccinia virus 7.5K promoter).

In bacterial systems, a number of expression vectors may be advantageously selected depending upon the use intended for the OXTR gene product being expressed. For example, when a large quantity of OXTR is to be produced, for the generation of pharmaceutical

compositions of OXTR or for raising antibodies to the OXTR protein, for example, vectors which direct the expression of high levels of fusion protein products that are readily purified may be desirable. Such vectors include, but are not limited to, the E. coli expression vector pUR278 (Ruther *et al.*, EMBO J. 2:1791, 1983), in which the OXTR coding sequence may be ligated individually into the vector in frame with the lacZ coding region so that a fusion protein is produced; pIN vectors (Inouye & Inouye, Nucleic Acids Res. 13:3101-3109, 1985; Van Heeke & Schuster, J. Biol. Chem. 264:5503-5509, 1989); and the like. PGEX vectors may also be used to express foreign polypeptides as fusion proteins with glutathione S-transferase (GST). In general, such fusion proteins are soluble and can easily be purified from lysed cells by adsorption to glutathione-agarose beads followed by elution in the presence of free glutathione. The PGEX vectors are designed to include thrombin or factor Xa protease cleavage sites so that the cloned target gene product can be released from the GST moiety.

In mammalian host cells, a number of viral-based expression systems may be utilized. In cases where an adenovirus is used as an expression vector, the OXTR nucleotide sequence of interest may be ligated to an adenovirus transcription/translation control complex, e.g., the late promoter and tripartite leader sequence. This chimeric gene may then be inserted in the adenovirus genome by *in vitro* or *in vivo* recombination. Insertion in a non-essential region of the viral genome (e.g., region E1 or E3) will result in a recombinant virus that is viable and capable of expressing the OXTR in infected hosts. (E.g., See Logan & Shenk, , Proc. Natl. Acad. Sci. USA 81:3655-3659, 1984).

For long-term, high-yield production of recombinant proteins, stable expression is preferred. For example, cell lines which stably express the OXTR sequence described above may be engineered. Rather than using expression vectors which contain viral origins of replication, host cells can be transformed with DNA controlled by appropriate expression control elements (e.g., promoter, enhancer sequences, transcription terminators, polyadenylation sites, etc.), and a selectable marker. Following the introduction of the foreign DNA, engineered cells may be allowed to grow for 1-2 days in an enriched media, and then are switched to a selective media. The selectable marker in the recombinant plasmid confers resistance to the selection and allows cells to stably integrate the plasmid into their chromosomes and grow to form foci which in turn can be cloned and expanded into cell lines. This method may

advantageously be used to engineer cell lines which express the OXTR. Such engineered cell lines may be particularly useful in screening and evaluation of compounds that affect the endogenous activity of the OXTR.

Alternatively, any fusion protein may be readily purified by utilizing an antibody specific for the fusion protein being expressed. For example, a system described by Janknecht *et al.* allows for the ready purification of non-denatured fusion proteins expressed in human cell lines (Janknecht, *et al.*, Proc. Natl. Acad. Sci. USA 88:8972-8976, 1991). In this system, the gene of interest is subcloned into a vaccinia recombination plasmid such that the gene's open reading frame is translationally fused to an amino-terminal tag consisting of six histidine residues. Extracts from cells infected with recombinant vaccinia virus are loaded onto Ni.2+. nitriloacetic acid-agarose columns and histidine-tagged proteins are selectively eluted with imidazole-containing buffers.

The OXTR protein can also be expressed in transgenic animals. Animals of any species, including, but not limited to, mice, rats, rabbits, guinea pigs, pigs, micro-pigs, goats, and non-human primates, for example, baboons, monkeys, and chimpanzees may be used to generate OXTR transgenic animals.

Any technique known in the art may be used to introduce the OXTR transgene into animals to produce the founder lines of transgenic animals. Such techniques include, but are not limited to pronuclear microinjection (U.S. Pat. No. 4,873,191); retrovirus mediated gene transfer into germ lines (Van der Putten *et al.*, Proc. Natl. Acad. Sci. USA 82:6148-6152, 1985); gene targeting in embryonic stem cells (Thompson *et al.*, Cell 56:313-321, 1989); electroporation of embryos (Lo, Mol. Cell. Biol. 3:1803-1814, 1983); and sperm-mediated gene transfer (Lavitrano *et al.*, Cell 57:717-723, 1989); etc. For a review of such techniques, see Gordon, Transgenic Animals, Intl. Rev. Cytol. 115:171-229, 1989, which is incorporated by reference herein in its entirety.

The present invention provides for transgenic animals that carry the OXTR transgene in all their cells, as well as animals which carry the transgene in some, but not all their cells, i.e., mosaic animals. The transgene may be integrated as a single transgene or in concatamers, e.g.,

head-to-head tandems or head-to-tail tandems. The transgene may also be selectively introduced into and activated in a particular cell type by following, for example, the teaching of Lasko *et al.* (Lasko, M. *et al.*, Proc. Natl. Acad. Sci. USA 89:6232-6236, 1992). Once transgenic animals have been generated, the expression of the recombinant OXTR gene may be assayed utilizing standard techniques. Initial screening may be accomplished by Southern blot analysis or PCR techniques to analyze animal tissues to assay whether integration of the transgene has taken place. The level of mRNA expression of the transgene in the tissues of the transgenic animals may also be assessed using techniques which include but are not limited to Northern blot analysis of tissue samples obtained from the animal, in situ hybridization analysis, and RT-PCR. Samples of OXTR gene-expressing tissue, may also be evaluated immunocytochemically using antibodies specific for the OXTR transgene product.

Antibodies to OXTR Proteins

Antibodies that specifically recognize one or more epitopes of OXTR or peptide fragments of the OXTR are also encompassed by the invention. Such antibodies include but are not limited to polyclonal antibodies, monoclonal antibodies (mAbs), humanized or chimeric antibodies, single chain antibodies, Fab fragments, F(ab').2 fragments, fragments produced by a Fab expression library, anti-idiotypic (anti-Id) antibodies, and epitope-binding fragments of any of the above.

The antibodies of the invention may be used, for example, in the detection of the OXTR in a biological sample and may, therefore, be utilized as part of a diagnostic or prognostic technique whereby patients may be tested for abnormal amounts of OXTR. Such antibodies may also be utilized in conjunction with, for example, compound screening schemes for the evaluation of the effect of test compounds on expression and/or activity of the OXTR gene product. Additionally, such antibodies can be used in conjunction with the gene therapy techniques described, for example, evaluate the normal and/or engineered OXTR-expressing cells prior to their introduction into the patient. Such antibodies may additionally be used as a method for the inhibition of abnormal OXTR activity. Thus, such antibodies may, therefore, be utilized as part of CAD treatment methods.

Methods of making and detecting labelled antibodies are well known (Campbell; Monoclonal

Antibody Technology, in: Laboratory Techniques in Biochemistry and Molecular Biology, Volume 13. Eds: Burdon R *et al.* Elsevier, Amsterdam (1984)). The term antibody includes both monoclonal antibodies, which are a substantially homogeneous population, and polyclonal antibodies which are heterogeneous populations. The term also includes inter alia, humanised and chimeric antibodies. Monoclonal antibodies to specific antigens may be obtained by methods known to those skilled in the art, such as from hybridoma cells, phage display libraries or other methods. Monoclonal antibodies may be inter alia, human, rat or mouse derived. For the production of human monoclonal antibodies, hybridoma cells may be prepared by fusing spleen cells from an immunised animal, e.g. a mouse, with a tumour cell. Appropriately secreting hybridoma cells may thereafter be selected (Koehler & Milstein, Nature 256:495-497 (1975); Cole *et al.*, "Monoclonal antibodies and Cancer Therapy", Alan R Liss Inc, New York N.Y. pp 77-96 (1985)). Such antibodies may be of any immunoglobulin class including IgG, IgM, IgE, IgA, IgD and any subclass thereof.

Polyclonal antibodies can be generated by immunisation of an animal (such as a mouse, rat, goat, horse, sheep etc) with OXTR.

Rodent antibodies may be humanised using recombinant DNA technology according to techniques known in the art. Alternatively, chimeric antibodies, single chain antibodies, Fab fragments may also be developed against the polypeptides of the invention (Huse *et al.*, Science 256:1275-1281 (1989)), using skills known in the art. Antibodies so produced have a number of uses which will be evident to the molecular biologist or immunologist skilled in the art. Such uses include, but are not limited to, monitoring enzyme expression, development of assays to measure enzyme activity and use as a therapeutic agent. Enzyme linked immunosorbant assays (ELISAs) are well known in the art and would be particularly suitable for detecting the OXTR or polypeptide fragments thereof in a test sample.

Screening Assay

The invention also provides a method for identifying modulators, i.e., test compounds (e.g., peptides, peptidomimetics, small molecules or other drugs) which modulate the activity of the OXTR gene or protein.

In one example, the invention provides methods for screening for test compounds for use in the treatment of CAD by screening for test compounds that modulate the activity of the OXTR protein, or a portion thereof. The test compounds of the present invention can be obtained using any of the numerous approaches in combinatorial library methods known in the art, including: biological libraries. The method can be a cell-based method or a cell free method. The screening methods according to the invention may be operated using conventional procedures, for example by bringing the test compound or compounds to be screened and an appropriate substrate into contact with the OXTR polypeptide, or a cell capable of producing it, or a cell membrane preparation thereof, and determining affinity for the OXTR polypeptide in accordance with standard techniques.

Any compound identified in this way may prove useful in the treatment of CAD in humans and/or other animals. The invention thus extends to a compound selected through its ability to regulate the activity of the OXTR protein *in vivo* as primarily determined in a screening assay utilising an OXTR polypeptide or a homologue or fragment thereof, or a gene coding therefore for use in the treatment of a disease in which the over- or under-activity or unregulated activity of the protein is implicated.

According to a further aspect of the invention there is provided a screening assay or method for identifying potential CAD therapeutic compounds comprising contacting an assay system capable of detecting the effect of a test compound against expression level of OXTR, with a test compound and assessing the change in expression level of OXTR. Compounds that modulate the expression of DNA or RNA of the OXTR polypeptide may be detected by a variety of assay systems. A suitable assay system may be a simple "yes/no" assay to determine whether there is a change in expression of a reporter gene, such as beta-galactosidase, luciferase, green fluorescent protein or others known to the person skilled in the art (reviewed by Naylor, Biochem. Pharmacol. 58:749-57, 1999). The assay system may be made quantitative by comparing the expression or function of a test sample with the levels of expression or function in a standard sample. Systems in which transcription factors are used to stimulate a positive output, such as transcription of a reporter gene, are generally referred to as "one-hybrid systems" (Wang, M.M. and Reed, R.R. Nature 364:121-126, 1993). Using a

transcription factor to stimulate a negative output (growth inhibition) may thus be referred to as a "reverse one-hybrid system" (Vidal et al, 1996, *supra*). Therefore, in an embodiment of the present invention, a reporter gene is placed under the control of the OXTR promoter.

In a further aspect of the invention a cell or cell line comprising a reporter gene under the control of the OXTR promoter is provided.

According to another aspect of the present invention there is provided a method of screening for a compound potentially useful for treatment of CAD which comprises assaying the compound for its ability to modulate the activity or amount of OXTR. Preferably the assay is selected from:

i) measurement of OXTR activity using a cell line which expresses the OXTR polypeptide or using purified OXTR polypeptide; and
ii) measurement of OXTR transcription or translation in a cell line expressing the OXTR polypeptide.

Thus, in a further aspect of the invention, cell cultures expressing the OXTR polypeptide can be used in a screen for therapeutic agents. Effects of test compounds may be assayed by changes in mRNA or protein of OXTR. As described above, cells (i.e. mammalian, bacterial, etc) can be engineered to express the OXTR polypeptide.

Thus, according to a further aspect of the invention there is provided a method of testing potential therapeutic agents for the ability to suppress the CAD phenotype comprising contacting a test compound with a cell engineered to express the OXTR polypeptide; and determining whether said test compound suppressed expression of the OXTR polypeptide.

We also provide a method for identifying inhibitors of transcription of OXTR, which method comprises contacting a potential therapeutic agent with a cell or cell line as described above and determining inhibition of OXTR transcription by the potential therapeutic agent by reference to a lack of or reduction in expression of the reporter gene.

Any convenient test compound or library of test compounds may be used in conjunction with the test assay. Particular test compounds include low molecular weight chemical compounds

(preferably with a molecular weight less than 1500 daltons) suitable as pharmaceutical or veterinary agents for human or animal use, or compounds for non-administered use such as cleaning/sterilising agents or for agricultural use. Test compounds may also be biological in nature, such as antibodies.

According to a further aspect of the invention there is provided a compound identified by a screening method as defined herein.

According to another aspect of the present invention there is provided use of a compound able to modulate the activity or amount of OXTR in the preparation of a medicament for the treatment of CAD. Modulation of the amount of OXTR by a compound may be brought about for example through altered gene expression level or message stability. Modulation of the activity of OXTR by a compound may also be brought about for example through compound binding to the OXTR protein. In one embodiment, modulation of OXTR comprises use of a compound able to reduce the activity or amount of OXTR. In another embodiment, modulation of OXTR comprises use of a compound able to increase the activity or amount of OXTR.

Diagnostic test

Methods for the diagnosis of CAD may, for example, utilize reagents such as the OXTR nucleotide sequences and OXTR antibodies. Specifically, such reagents may be used, for example, for: (1) the detection of the presence of OXTR gene mutations, or the detection of either over- or under-expression of OXTR mRNA relative to a control; (2) the detection of either an over- or an under-abundance of OXTR gene product relative to a control; and (3) the detection of perturbations or abnormalities in the signal transduction pathway mediated by OXTR.

The methods described herein may be performed, for example, by utilizing pre-packaged diagnostic kits comprising at least one specific OXTR nucleotide sequence or OXTR antibody reagent described herein, which may be conveniently used, e.g., in clinical settings, to diagnose patients exhibiting body weight disorder abnormalities.

For the detection of OXTR mutations, any nucleated cell can be used as a starting source for genomic nucleic acid. For the detection of OXTR gene expression or OXTR gene products, any cell type or tissue in which the OXTR gene is expressed can be used.

Detection of OXTR polymorphisms

Knowledge of polymorphisms can be of assistance in identifying patients susceptible to particular diseases and those most suited to therapy with particular pharmaceutical agents (the latter is often termed "pharmacogenetics"). Pharmacogenetics can also be used in pharmaceutical research to assist the drug selection process. Polymorphisms are used in mapping the human genome and to elucidate the genetic component of diseases. The reader is directed to the following references for background details on pharmacogenetics and other uses of polymorphism detection: Linder *et al.* (1997), Clinical Chemistry, **43**, 254; Marshall (1997), Nature Biotechnology, **15**, 1249; International Patent Application WO 97/40462, Spectra Biomedical; and Schafer *et al.* (1998), Nature Biotechnology, **16**, 33.

The present inventors have identified a number of polymorphisms within and around the OXTR gene with demonstrable genetic association to coronary artery disease (CAD). The type and position of polymorphisms, which are identified in Table 3, are identified relative to the start of nucleotide #1 of exon 1 according to the position in Genbank ID:NM 000916. Tables 4 and 5 provide sequence adjacent to the polymorphism site, which sequence can be used to unambiguously locate the position of the polymorphism in a nucleic acid sample. SEQ ID Nos: 3 – 9 provide sequence of the polymorphism and sequence adjacent the polymorphism.

According to one aspect of the present invention there is provided a method for the diagnosis of a nucleotide polymorphism associated with CAD, which method comprises determining the sequence of the nucleotide at position 31 of any one of SEQ ID NOs: 3 to 9, and determining the status of the human by reference to polymorphism(s) detected.

The term human includes both a human having or suspected of having inflammatory bowel disease and an asymptomatic human who may be tested for predisposition or susceptibility to

CAD. At each position the human may be homozygous for an allele or the human may be a heterozygote.

In one embodiment of the invention preferably the method for diagnosis described herein is one in which the single nucleotide polymorphism at position 31 (according to SEQ ID NO: 3) is the presence of C and/or A.

In another embodiment of the invention preferably the method of diagnosis described herein is one in which the single nucleotide polymorphism at position 31 (according to SEQ ID NO: 4) is the presence of A and/or G.

In another embodiment of the invention preferably the method of diagnosis described herein is one in which the single nucleotide polymorphism at position 31 (according to SEQ ID NO: 5) is the presence of T and/or A.

In another embodiment of the invention preferably the method of diagnosis described herein is one in which the single nucleotide polymorphism at position 31 (according to SEQ ID NO: 6) is the presence of T and/or A.

In another embodiment of the invention preferably the method of diagnosis described herein is one in which the single nucleotide polymorphism at position 31 (according to SEQ ID NO: 7) is the presence of C and/or T.

In another embodiment of the invention preferably the method of diagnosis described herein is one in which the single nucleotide polymorphism at position 31 (according to SEQ ID NO: 8) is the presence of G and/or A.

In another embodiment of the invention preferably the method of diagnosis described herein is one in which the single nucleotide polymorphism at position 31 (according to SEQ ID NO: 9) is the presence of T and/or A.

The method for diagnosis is preferably one in which the sequence is determined by a method selected from amplification refractory mutation system and restriction fragment length polymorphism.

In another aspect of the invention we provide a method for the diagnosis or prognosis or inflammatory bowel disease, which method comprises:

i) obtaining sample nucleic acid from an individual,

ii) detecting the presence or absence of a variant nucleotide at position 31 relative to one or more of the sequences disclosed in SEQ ID NO: 3 to 9; and,

iii) determining the status of the individual by reference to the particular variant polymorphism.

The test sample of nucleic acid is conveniently a sample of blood, bronchoalveolar lavage fluid, sputum, or other body fluid or tissue obtained from an individual. It will be appreciated that the test sample may equally be a nucleic acid sequence corresponding to the sequence in the test sample, that is to say that all or a part of the region in the sample nucleic acid may firstly be amplified using any convenient technique e.g. PCR, before use in the analysis of OXTR variation.

It will be apparent to the person skilled in the art that there are a large number of analytical procedures, which may be used to detect the presence or absence of the various SNPs of the invention. In general, the detection of allelic variation requires a mutation discrimination technique, optionally an amplification reaction and a signal generation system. Table 1 lists a number of mutation detection techniques, some based on the PCR. These may be used in combination with a number of signal generation systems, a selection of which is listed in List 1. Further amplification techniques are listed in List 2. Many current methods for the detection of allelic variation are reviewed by Nollau *et al.*, Clin. Chem. 43, 1114-1120, 1997; and in standard textbooks, for example "Laboratory Protocols for Mutation Detection", Ed. by U. Landegren, Oxford University Press, 1996 and "PCR", 2nd Edition by Newton & Graham, BIOS Scientific Publishers Limited, 1997.

Abbreviations:

ALEX™	Amplification refractory mutation system linear extension
APEX	Arrayed primer extension
ARMS™	Amplification refractory mutation system
b-DNA	Branched DNA
CMC	Chemical mismatch cleavage
Bp	base pair
COPS	Competitive oligonucleotide priming system
DGGE	Denaturing gradient gel electrophoresis
FRET	Fluorescence resonance energy transfer
LCR	Ligase chain reaction
MASDA	Multiple allele specific diagnostic assay
NASBA	Nucleic acid sequence based amplification
OLA	Oligonucleotide ligation assay
PCR	Polymerase chain reaction
PTT	Protein truncation test
RFLP	Restriction fragment length polymorphism
SDA	Strand displacement amplification
SNP	Single nucleotide polymorphism
SSCP	Single-strand conformation polymorphism analysis
SSR	Self sustained replication
TGGE	Temperature gradient gel electrophoresis

Table 1 - Mutation Detection Techniques

General: DNA sequencing, Sequencing by hybridisation

Scanning: PTT*, SSCP, DGGE, TGGE, Cleavase, Heteroduplex analysis, CMC, Enzymatic mismatch cleavage

* Note: not useful for detection of promoter polymorphisms.

Hybridisation Based

Solid phase hybridisation: Dot blots, MASDA, Reverse dot blots, Oligonucleotide arrays (DNA Chips)

Solution phase hybridisation: Taqman™ - US-5210015 & US-5487972 (Hoffmann-La Roche), Molecular Beacons - Tyagi *et al* (1996), Nature Biotechnology, **14**, 303; WO 95/13399 (Public Health Inst., New York)

Extension Based: ARMS™, ALEX™ - European Patent No. EP 332435 B1 (Zeneca Limited), COPS - Gibbs *et al* (1989), Nucleic Acids Research, **17**, 2347.

Incorporation Based: Mini-sequencing, APEX

Restriction Enzyme Based: RFLP, Restriction site generating PCR

Ligation Based: OLA

Other: Invader assay

List 1 - Signal Generation or Detection Systems

Fluorescence: FRET, Fluorescence quenching, Fluorescence polarisation - United Kingdom Patent No. 2228998 (Zeneca Limited)

Other: Chemiluminescence, Electrochemiluminescence, Raman, Radioactivity, Colorimetric, Hybridisation protection assay, Mass spectrometry

List 2 - Further Amplification Methods

SSR, NASBA, LCR, SDA, b-DNA

Preferred mutation detection techniques include ARMS™, ALEX™, COPS, Taqman, Molecular Beacons, RFLP, and restriction site based PCR and FRET techniques.

Particularly preferred methods include Taqman, ARMS™ and RFLP based methods. Taqman is an especially preferred method.

Assays, for example reporter-based assays, may be devised to detect whether one or more of the above polymorphisms affect transcription levels.

In a further aspect, the diagnostic methods of the invention, are used to assess the predisposition and/or susceptibility of an individual to CAD, and the present invention may be used to recognise individuals who are particularly at risk from developing CAD conditions.

In a further aspect, the diagnostic methods of the invention are used in the development of new drug therapies, which selectively target one or more allelic variants identified herein. Identification of a link between a particular allelic variant and predisposition to disease development or response to drug therapy may have a significant impact on the design of new drugs. Drugs may be designed to regulate the biological activity of variants implicated in the disease process whilst minimising effects on other variants.

In a further diagnostic aspect of the invention the presence or absence of variant nucleotides is detected by reference to the loss or gain of, optionally engineered, sites recognised by restriction enzymes. The person of ordinary skill will be able to design and implement diagnostic procedures based on the detection of restriction fragment length polymorphism due to the loss or gain of one or more of the sites.

The invention further provides nucleotide primers, which detect the polymorphisms of the invention.

The invention further provides nucleotide probes, which can detect the polymorphisms of the invention.

According to another aspect of the present invention a person of ordinary skill will be able to design allele specific primers or probes capable of detecting a polymorphism at position 31 at one or more of: SEQ ID NO: 3 to 9.

An allele specific primer is used, generally together with a constant primer, in an amplification reaction such as a PCR reaction, which provides the discrimination between alleles through selective amplification of one allele at a particular sequence position e.g. as used for ARMS™ assays. The allele specific primer is preferably 17- 50 nucleotides, more preferably about 17-35 nucleotides, more preferably about 17-30 nucleotides.
An allele specific primer preferably corresponds exactly with the allele to be detected but derivatives thereof are also contemplated wherein about 6-8 of the nucleotides at the 3' terminus correspond with the allele to be detected and wherein up to 10, such as up to 8, 6, 4,

2, or 1 of the remaining nucleotides may be varied without significantly affecting the properties of the primer.

Primers may be manufactured using any convenient method of synthesis. Examples of such methods may be found in standard textbooks, for example "Protocols for Oligonucleotides and Analogues; Synthesis and Properties," Methods in Molecular Biology Series; Volume 20; Ed. Sudhir Agrawal, Humana ISBN: 0-89603-247-7; 1993; 1st Edition. If required the primer(s) may be labelled to facilitate detection.

According to another aspect of the present invention there is provided an allele-specific oligonucleotide probe capable of detecting a polymorphism at position 31 of any one of SEQ ID NOs: 3 to 9.

The allele-specific oligonucleotide probe is preferably 17- 50 nucleotides, more preferably about 17-35 nucleotides, more preferably about 17-30 nucleotides. Representative examples of oligonucleotide probes are those identified in SEQ ID NO: 3 to 9, but wherein the nucleotide at position 31 is one or other of the polymorphic alleles.

The design of such probes will be apparent to the molecular biologist of ordinary skill. Such probes are of any convenient length such as up to 50 bases, up to 40 bases, more conveniently up to 30 bases in length, such as for example 8-25 or 8-15 bases in length. In general such probes will comprise base sequences entirely complementary to the corresponding wild type or variant locus in the gene. However, if required one or more mismatches may be introduced, provided that the discriminatory power of the oligonucleotide probe is not unduly affected. The probes of the invention may carry one or more labels to facilitate detection. According to another aspect of the present invention there is provided a diagnostic kit comprising a diagnostic primer of the invention and/or an allele-specific oligonucleotide primer of the invention.

The diagnostic kits may comprise appropriate packaging and instructions for use in the methods of the invention. Such kits may further comprise appropriate buffer(s) and polymerase(s) such as thermostable polymerases, for example taq polymerase.

In another aspect of the invention, the single nucleotide polymorphisms of this invention may be used as genetic markers for this region in linkage studies.

Detection of the CAD Gene and Transcripts

OXTR gene transcripts and mutations within the OXTR gene can be detected by utilizing a number of techniques. Nucleic acid from any nucleated cell can be used as the starting point for such assay techniques, and may be isolated according to standard nucleic acid preparation procedures, which are well known to those of skill in the art.

DNA may be used in hybridization or amplification assays of biological samples to detect abnormalities involving OXTR gene structure or gene regulatory elements, including point mutations, insertions, deletions and chromosomal rearrangements. Such assays may include, but are not limited to, Southern analyses, single stranded conformational polymorphism analyses (SSCP), and PCR analyses.

Such diagnostic methods for the detection of OXTR gene-specific mutations can involve for example, contacting and incubating nucleic acids including recombinant DNA molecules, cloned genes or degenerate variants thereof, obtained from a sample, for example, derived from a patient sample or other appropriate cellular source, with one or more labeled nucleic acid reagents including recombinant DNA molecules, under conditions favorable for the specific annealing of these reagents to their complementary sequences within the OXTR gene. Preferably, the lengths of these nucleic acid reagents are at least 15 to 30 nucleotides. After incubation, all non-annealed nucleic acids are removed from the nucleic acid: OXTR molecule hybrid. The presence of nucleic acids which have hybridized, if any such molecules exist, is then detected. Using such a detection scheme, the nucleic acid from the cell type or tissue of interest can be immobilized, for example, to a solid support such as a membrane, or a plastic surface such as that on a microtiter plate or polystyrene beads. Detection of the remaining, annealed, labeled OXTR nucleic acid reagents is accomplished using standard techniques well-known to those in the art. The OXTR gene sequences to which the nucleic acid reagents have annealed can be compared to the annealing pattern expected from a normal OXTR gene sequence in order to determine whether an OXTR gene mutation is present.

Detection of the OXTR Gene Products

Antibodies directed against wild type or mutant OXTR gene products or conserved variants or peptide fragments thereof, can also be used as CAD diagnostics. Such diagnostic methods, can be used to detect abnormalities in the level of OXTR gene expression, or abnormalities in the structure and/or temporal, tissue, cellular, or subcellular location of the OXTR, and may be performed *in vivo* or *in vitro*, such as, for example, on biopsy tissue.

For example, antibodies directed to epitopes of the OXTR can be used *in vivo* to detect the pattern and level of expression of the OXTR in the body. Such antibodies can be labeled, and injected into a subject in order to visualize binding to the OXTR expressed in the body using methods such as X-rays, CAT-scans, or MRI.

Additionally, any OXTR fusion protein or OXTR conjugated protein whose presence can be detected, can be administered. For example, OXTR fusion or conjugated proteins labeled with a radio-opaque or other appropriate compound can be administered and visualized *in vivo*, as discussed, above for labeled antibodies.

Alternatively, immunoassays or fusion protein detection assays, as described above, can be utilized on biopsy and autopsy samples *in vitro* to permit assessment of the expression pattern of the OXTR.

The tissue or cell type to be analyzed will generally include those which are known, or suspected, to express the OXTR gene. The protein isolation methods employed herein can, for example, be such as those described in Harlow and Lane (Harlow, E. and Lane, D, Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1989), which is incorporated herein by reference in its entirety. The isolated cells can be derived from cell culture or from a patient. The analysis of cells taken from culture may be a necessary step in the assessment of cells that could be used as part of a cell-based gene therapy technique or, alternatively, to test the effect of compounds on the expression of the OXTR gene.

Gene therapy

The invention encompasses gene therapy methods and compositions for treating and preventing CAD.

In one example, the loss of normal OXTR gene product function can result in the development of CAD. Increasing OXTR gene product activity, or activation of the OXTR pathway (e.g., downstream activation) would therefore facilitate progress in individuals exhibiting a deficient level of OXTR gene expression and/or OXTR activity.

Alternatively, CAD may be ameliorated by decreasing the level of OXTR gene expression, and/or OXTR gene activity, and/or downregulating activity of the OXTR pathway (e.g., by targeting downstream signaling events). Different approaches are discussed below.

Inhibition of OXTR Expression or OXTR Activity

Any method that neutralizes or inhibits expression of the OXTR gene (either transcription or translation) can be used to prevent or treat CAD.

For example, the administration of soluble peptides, proteins, fusion proteins, or antibodies (including anti-idiotypic antibodies) that bind to and "neutralize" circulating OXTR can be used. Such OXTR neutralizing peptides, proteins, fusion proteins, anti-idiotypic antibodies or Fabs are administered to a subject in amounts sufficient to treat or prevent CAD.

In an alternate embodiment, therapy can be designed to reduce the level of endogenous OXTR gene expression, for example, using antisense or ribozyme approaches to inhibit or prevent translation of OXTR mRNA-transcripts; triple helix approaches to inhibit transcription of the OXTR gene; or targeted homologous recombination to inactivate or "knock out" the OXTR gene or its endogenous promoter. Antisense, ribozyme or DNA constructs can be administered directly to the site containing the target cells; or can be directed to the target cells.

Antisense approaches involve the design of oligonucleotides (either DNA or RNA) that are complementary to OXTR mRNA. The antisense oligonucleotides will bind to the complementary OXTR mRNA transcripts and prevent translation. Absolute complementarity, although preferred, is not required. A sequence "complementary" to a portion of an RNA, as referred to herein, means a sequence having sufficient complementarity to be able to hybridize with the RNA, forming a stable duplex; in the case of double-stranded antisense nucleic acids, a single strand of the duplex DNA may thus be tested, or triplex formation may be assayed. The ability to hybridize will depend on both the degree of complementarity and the length of the antisense nucleic acid. Generally, the longer the hybridizing nucleic acid, the more base mismatches with an RNA it may contain and still form a stable duplex (or triplex, as the case may be). One skilled in the art can ascertain a tolerable degree of mismatch by use of standard procedures to determine the melting point of the hybridized complex.

Oligonucleotides that are complementary to the 5' end of the message, for example, the 5' untranslated sequence up to and including the AUG initiation codon, should work most efficiently at inhibiting translation. However, sequences complementary to the 3' untranslated sequences of mRNAs have been shown to be effective at inhibiting translation of mRNAs as well. Thus, oligonucleotides complementary to either the 5'- or 3'-non-translated, non-coding regions of the OXTR can be used in an antisense approach to inhibit translation of endogenous OXTR mRNA. Oligonucleotides complementary to the 5' untranslated region of the mRNA should include the complement of the AUG start codon. Antisense oligonucleotides complementary to mRNA coding regions are less efficient inhibitors of translation but could be used in accordance with the invention. Whether designed to hybridize to the 5'-, 3'- or coding region of OXTR mRNA, antisense nucleic acids should be at least six nucleotides in length, and are preferably oligonucleotides ranging from 6 to about 50 nucleotides in length. In specific aspects, the oligonucleotide is at least 10 nucleotides, at least 17 nucleotides, at least 25 nucleotides, or at least 50 nucleotides in length.

Regardless of the choice of target sequence, it is preferred that *in vitro* studies are first performed to quantitate the ability of the antisense oligonucleotide to inhibit gene expression. It is preferred that these studies utilize controls that distinguish between antisense gene

inhibition and nonspecific biological effects of oligonucleotides. It is also preferred that these studies compare levels of the target RNA or protein with that of an internal control RNA or protein. Additionally, it is envisioned that results obtained using the antisense oligonucleotide are compared with those obtained using a control oligonucleotide. It is preferred that the control oligonucleotide is of approximately the same length as the test oligonucleotide and that the nucleotide sequence of the oligonucleotide differs from the antisense sequence no more than is necessary to prevent specific hybridization to the target sequence.

While antisense nucleotides complementary to the OXTR coding region sequence could be used, those complementary to the transcribed untranslated region are most preferred. The antisense molecules should be delivered to cells which express the OXTR *in vivo*. A number of methods have been developed for delivering antisense DNA or RNA to cells; for example, antisense molecules can be injected directly into the tissue site, or modified antisense molecules, designed to target the desired cells (e.g., antisense linked to peptides or antibodies that specifically bind receptors or antigens expressed on the target cell surface) can be administered systemically.

Ribozyme molecules designed to catalytically cleave OXTR mRNA transcripts can also be used to prevent translation of OXTR mRNA and expression of OXTR. (See, e.g., WO90/11364; Sarver *et al.*, Science 247:1222-1225, 1990). While ribozymes that cleave mRNA at site specific recognition sequences can be used to destroy OXTR mRNAs, the use of hammerhead ribozymes is preferred. Hammerhead ribozymes cleave mRNAs at locations dictated by flanking regions that form complementary base pairs with the target mRNA. The sole requirement is that the target mRNA have the following sequence of two bases: 5'-UG-3'. The construction and production of hammerhead ribozymes is well known in the art and is described more fully in Haseloff and Gerlach, Nature 334:585-591, 1988. There are hundreds of potential hammerhead ribozyme cleavage sites within the nucleotide sequence of human OXTR cDNA. Preferably the ribozyme is engineered so that the cleavage recognition site is located near the 5' end of the OXTR mRNA; i.e., to increase efficiency and minimize the intracellular accumulation of non-functional mRNA transcripts.

The ribozymes of the present invention also include RNA endoribonucleases (hereinafter

"Cech-type ribozymes") such as the one which occurs naturally in Tetrahymena Thermophila (known as the IVS, or L-19 IVS RNA) and which has been extensively described by Thomas Cech and collaborators (Zaug, *et al.*, Science 224:574-578, 1984; Zaug and Cech, Science 231:470-475, 1986; Zaug, *et al.*, Nature 324:429-433, 1986; patent application No. WO 88/04300; Been and Cech, Cell 47:207-216, 1986). The Cech-type ribozymes have an eight basepair active site that hybridizes to a target RNA sequence, whereafter cleavage of the target RNA takes place. The invention encompasses those Cech-type ribozymes that target eight basepair active site sequences that are present in OXTR.

As in the antisense approach, the ribozymes can be composed of modified oligonucleotides (e.g. for improved stability, targeting, etc.) and should be delivered to cells which express the OXTR *in vivo*. A preferred method of delivery involves using a DNA construct "encoding" the ribozyme under the control of a strong constitutive pol III or pol II promoter, so that transfected cells will produce sufficient quantities of the ribozyme to destroy endogenous OXTR messages and inhibit translation. Because ribozymes, unlike antisense molecules, are catalytic, a lower intracellular concentration is required for efficiency.

In an alternative embodiment for neutralizing circulating OXTR, cells that are genetically engineered to express such soluble or secreted forms of OXTR can be administered to a patient, whereupon they will serve as "bioreactors" *in vivo* to provide a continuous supply of the neutralizing protein.

Endogenous OXTR gene expression can also be reduced by inactivating or "knocking out" the OXTR gene or its promoter using targeted homologous recombination. (E.g., see Smithies *et al.*, Nature 317:230-234, 1985; Thomas & Capecchi, Cell 51:503-512, 1987; Thompson *et al.*, Cell 5:313-321, 1989; each of which is incorporated by reference herein in its entirety).

In yet another embodiment of the invention, the activity of OXTR can be reduced using a "dominant negative" approach to effectuate OXTR. To this end, constructs that encode defective OXTRs can be used in gene therapy approaches to diminish the activity of the OXTR in appropriate target cells.

Restoration or Increase in OXTR Expression or Activity

With respect to an increase in the level of OXTR gene expression and/or OXTR gene product activity, OXTR nucleic acid sequences can be utilized for the treatment of CAD. Where the cause of CAD is a defective OXTR gene, treatment can be administered, for example, in the form of gene replacement therapy. Specifically, one or more copies of a normal OXTR gene or a portion of the OXTR gene that directs the production of an OXTR gene product exhibiting normal function, can be inserted into the appropriate cells within a patient or animal subject, using vectors which include, but are not limited to, adenovirus, adeno-associated virus, retrovirus, and herpes virus vectors, in addition to other particles that introduce DNA into cells, such as liposomes.

Additional methods that can be utilized to increase the overall level of OXTR gene expression and/or OXTR activity include the introduction of appropriate OXTR-expressing cells, preferably autologous cells, into a patient at positions and in numbers which are sufficient to ameliorate the symptoms of CAD. Such cells can be either recombinant or non-recombinant. Among the cells that can be administered to increase the overall level of OXTR gene expression in a patient are normal cells which express the OXTR gene. The cells can be administered to the site of interest. Such cell-based gene therapy techniques are well known to those skilled in the art, see, for example, Anderson, *et al.*, U.S. Pat. No. 5,399,349; Mulligan & Wilson, U.S. Pat. No. 5,460,959.

Finally, compounds, identified in the assays described above, that stimulate or enhance the signal transduced by activated OXTR, for example, by activating downstream signalling proteins in the OXTR cascade, and thereby by-passing the defective OXTR. The formulation and mode of administration will depend upon the physico-chemical properties of the compound.

Pharmaceutical composition containing the Identified Candidate Compound

The compounds having the desired activity may be administered in a physiologically acceptable carrier to a CAD patient. Such compositions of the invention may be in a form suitable for oral use (for example as tablets, lozenges, hard or soft capsules, aqueous or oily

suspensions, emulsions, dispersible powders or granules, syrups or elixirs), for topical use (for example as creams, ointments, gels, or aqueous or oily solutions or suspensions), for administration by inhalation (for example as a finely divided powder or a liquid aerosol), for administration by insufflation (for example as a finely divided powder) or for parenteral administration (for example as a sterile aqueous or oily solution for intravenous, subcutaneous, intramuscular or intramuscular dosing or as a suppository for rectal dosing).

The compositions of the invention may be obtained by conventional procedures using conventional pharmaceutical excipients, well known in the art. Thus, compositions intended for oral use may contain, for example, one or more colouring, sweetening, flavouring and/or preservative agents.

Suitable pharmaceutically acceptable excipients for a tablet formulation include, for example, inert diluents such as lactose, sodium carbonate, calcium phosphate or calcium carbonate, granulating and disintegrating agents such as corn starch or algenic acid; binding agents such as starch; lubricating agents such as magnesium stearate, stearic acid or talc; preservative agents such as ethyl or propyl p-hydroxybenzoate, and anti-oxidants, such as ascorbic acid. Tablet formulations may be uncoated or coated either to modify their disintegration and the subsequent absorption of the active ingredient within the gastrointestinal track, or to improve their stability and/or appearance, in either case, using conventional coating agents and procedures well known in the art.

Compositions for oral use may be in the form of hard gelatin capsules in which the active ingredient is mixed with an inert solid diluent, for example, calcium carbonate, calcium phosphate or kaolin, or as soft gelatin capsules in which the active ingredient is mixed with water or an oil such as peanut oil, liquid paraffin, or olive oil.

Aqueous suspensions generally contain the active ingredient in finely powdered form together with one or more suspending agents, such as sodium carboxymethylcellulose, methylcellulose, hydroxypropylmethylcellulose, sodium alginate, polyvinyl-pyrrolidone, gum tragacanth and gum acacia; dispersing or wetting agents such as lecithin or condensation products of an alkylene oxide with fatty acids (for example polyoxethylene stearate), or

condensation products of ethylene oxide with long chain aliphatic alcohols, for example heptadecaethyleneoxycetanol, or condensation products of ethylene oxide with partial esters derived from fatty acids and a hexitol such as polyoxyethylene sorbitol monooleate, or condensation products of ethylene oxide with long chain aliphatic alcohols, for example heptadecaethyleneoxycetanol, or condensation products of ethylene oxide with partial esters derived from fatty acids and a hexitol such as polyoxyethylene sorbitol monooleate, or condensation products of ethylene oxide with partial esters derived from fatty acids and hexitol anhydrides, for example polyethylene sorbitan monooleate. The aqueous suspensions may also contain one or more preservatives (such as ethyl or propyl p-hydroxybenzoate, anti-oxidants (such as ascorbic acid), colouring agents, flavouring agents, and/or sweetening agents (such as sucrose, saccharine or aspartame).

Oily suspensions may be formulated by suspending the active ingredient in a vegetable oil (such as arachis oil, olive oil, sesame oil or coconut oil) or in a mineral oil (such as liquid paraffin). The oily suspensions may also contain a thickening agent such as beeswax, hard paraffin or cetyl alcohol. Sweetening agents such as those set out above, and flavouring agents may be added to provide a palatable oral preparation. These compositions may be preserved by the addition of an anti-oxidant such as ascorbic acid.

Dispersible powders and granules suitable for preparation of an aqueous suspension by the addition of water generally contain the active ingredient together with a dispersing or wetting agent, suspending agent and one or more preservatives. Suitable dispersing or wetting agents and suspending agents are exemplified by those already mentioned above. Additional excipients such as sweetening, flavouring and colouring agents, may also be present.

The pharmaceutical compositions of the invention may also be in the form of oil-in-water emulsions. The oily phase may be a vegetable oil, such as olive oil or arachis oil, or a mineral oil, such as for example liquid paraffin or a mixture of any of these. Suitable emulsifying agents may be, for example, naturally-occurring gums such as gum acacia or gum tragacanth, naturally-occurring phosphatides such as soya bean, lecithin, an esters or partial esters derived from fatty acids and hexitol anhydrides (for example sorbitan monooleate) and condensation products of the said partial esters with ethylene oxide such as polyoxyethylene

sorbitan monooleate. The emulsions may also contain sweetening, flavouring and preservative agents.

Syrups and elixirs may be formulated with sweetening agents such as glycerol, propylene glycol, sorbitol, aspartame or sucrose, and may also contain a demulcent, preservative, flavouring and/or colouring agent.

The pharmaceutical compositions may also be in the form of a sterile injectable aqueous or oily suspension, which may be formulated according to known procedures using one or more of the appropriate dispersing or wetting agents and suspending agents, which have been mentioned above. A sterile injectable preparation may also be a sterile injectable solution or suspension in a non-toxic parenterally-acceptable diluent or solvent, for example a solution in 1,3-butanediol.

Suppository formulations may be prepared by mixing the active ingredient with a suitable non-irritating excipient which is solid at ordinary temperatures but liquid at the rectal temperature and will therefore melt in the rectum to release the drug. Suitable excipients include, for example, cocoa butter and polyethylene glycols.

Topical formulations, such as creams, ointments, gels and aqueous or oily solutions or suspensions, may generally be obtained by formulating an active ingredient with a conventional, topically acceptable, vehicle or diluent using conventional procedure well known in the art.

Compositions for administration by insufflation may be in the form of a finely divided powder containing particles of average diameter of, for example, 30μ or much less, the powder itself comprising either active ingredient alone or diluted with one or more physiologically acceptable carriers such as lactose. The powder for insufflation is then conveniently retained in a capsule containing, for example, 1 to 50mg of active ingredient for use with a turbo-inhaler device, such as is used for insufflation of the known agent sodium cromoglycate.

Compositions for administration by inhalation may be in the form of a conventional pressurised aerosol arranged to dispense the active ingredient either as an aerosol containing finely divided solid or liquid droplets. Conventional aerosol propellants such as volatile fluorinated hydrocarbons or hydrocarbons may be used and the aerosol device is conveniently arranged to dispense a metered quantity of active ingredient.

For further information on Formulation the reader is referred to Chapter 25.2 in Volume 5 of Comprehensive Medicinal Chemistry (Corwin Hansch; Chairman of Editorial Board), Pergamon Press 1990.

The amount of active ingredient that is combined with one or more excipients to produce a single dosage form will necessarily vary depending upon the host treated and the particular route of administration. For example, a formulation intended for oral administration to humans will generally contain, for example, from 0.5 mg to 2 g of active agent compounded with an appropriate and convenient amount of excipients which may vary from about 5 to about 98 percent by weight of the total composition. Dosage unit forms will generally contain about 1 mg to about 500 mg of an active ingredient. For further information on Routes of Administration and Dosage Regimes the reader is referred to Chapter 25.3 in Volume 5 of Comprehensive Medicinal Chemistry (Corwin Hansch; Chairman of Editorial Board), Pergamon Press 1990.

The size of the dose for therapeutic or prophylactic purposes of a compound will naturally vary according to the nature and severity of the conditions, the age and sex of the animal or patient and the route of administration, according to well known principles of medicine.

In using a compound for therapeutic or prophylactic purposes it will generally be administered so that a daily dose in the range, for example, 0.5 mg to 75 mg per kg body weight is received, given if required in divided doses. In general lower doses will be administered when a parenteral route is employed. Thus, for example, for intravenous administration, a dose in the range, for example, 0.5 mg to 30 mg per kg body weight will generally be used. Similarly, for administration by inhalation, a dose in the range, for

example, 0.5 mg to 25 mg per kg body weight will be used. Oral administration is however preferred.

It will be obvious to those skilled in the art to which the invention pertains, that various changes and modifications may be made without departing from the scope of the invention defined by the claims.

Examples

Example 1

530 affected sib-pairs (ASPs) from 407 families with both sibs having CAD before 65 years were collected from hospitals in Sweden, Italy and Germay. All ASPs were recruited either from hospital discharge databases, existing research-based patient databases or from coronary care units.

Probands were individuals with documented myocardial infarction (MI) or other symptomatic acute coronary syndrome (ACS; defined as hospitalisation for one of the following indications: a) unstable angina; b) thrombolysis for suspected MI (localised ST-elevation in 2 or more ECG leads); c) emergency revascularisation for control of typical ischaemic chest pain at rest) first diagnosed before 65 years and confirmed by the physician. Affected sibs were individuals with confirmed MI, other symptomatic ACS as for the proband but in addition, chronic stable angina first diagnosed before 65 years and confirmed by physician, was included.

Preparation of genomic DNA from blood cells

Blood samples were stored at -80°C. Before extraction the samples were thawed at 37°C in a water bath. To extract the genomic DNA, using the PUREGENE DNA Isolation kit from Gentra Systems, the blood cells were added to 30ml of RBC lysis solution in a 50ml centrifuge tube. The mixture was inverted twice while incubating for 10minutes (min) at room temperature (RT). To separate the white cells from other lysed material, the mixture was centrifuged in a RT-7™ from Sorvall Ltd. at 2,000X g for10min. Most of the supernatant was removed leaving approximately 100ml of residual solution to resuspend the cells in by vigorously votexing. Following resuspension 10ml of cell lysis solution were added and mixed by pipetting. To digest any RNA, 50ml of RNase A solution were added to the lysate, which was then incubated at 37°C for 15min. After RNA digestion, 3.3ml of protein

precipitation solution was added and the mixture was vortexed for 20seconds (s). To separate the proteins from the DNA, the mixture was again centrifuged at 2,000X g for 10min. The supernatant retaining the DNA was then poured off into another 50ml centrifuge tube containing 10ml of 100% isopropanol and mixed by inverting 50X. To separate the DNA from the aqueous solution, it was removed with an inoculating loop and washed in 10ml 70% ethanol in a 50ml centrifuge tube. to remove any residual salt. The DNA was removed from the 70% ethanol using an inoculating loop and air dried briefly. To hydrate, the DNA was added to1ml of DNA hydration solution in a 14ml centrifuge tube and this was rocked at RT overnight allowing the DNA to equilibrate. The resulting DNA samples were transferred to 1.5ml tubes and stored at -80°C. The concentration of extracted genomic DNA was quantified using the Fluorocount™ from Packard Instruments Co. The DNA was diluted to 4 ng/ml into a working plate.

Dispensing DNA from a working plate into several 96-well plates for subsequent polymerase chain reaction (PCR) amplification reactions was performed using the Hydra™ 96 from Robbins Scientific, Inc. The Hydra™ was programmed to dispense 5ml of DNA from each well of a 'working plate' into the corresponding wells of the required number of PCR plates. Before dispensing, the Hydra™ was washed with water. Following dispensing the Hydra™ was sterilised by washing with water, with 2% chloros industrial, and again with water. The resulting PCR plates, containing 20ng of DNA in each well, were dried at 80°C for 1hr. The plates were stored at -20°C.

Microsatellite genotyping for genome scan

A total of 384 microsatellite markers were genotyped across the CAD ASP families. The majority of these were from the linkage mapping set 2.0 (Applied Biosystems). Additional markers were included to replace those excluded by error checking procedures. The amplification of microsatellite markers by PCR and subsequent pooling of the amplified products into panels were carried out using a RapidGene™ automated system from Oxagen Ltd. The PCR plates contained 20ng of dried genomic DNA in each well. The 1.5ml tubes contained 5mM of appropriate 'forward' oligonucleotide, 5mM of the corresponding 'reverse' oligonucleotide, 1X PCR buffer II, 2.5mM MgCl2, 1mM dNTP, and 0.06u/ml AmpliTaq Gold™ DNA polymerase in final volume of 1.2ml. During the run, 10ml of the appropriate

PCR reaction mixture were dispensed into the wells of the plates. Following amplification the reactions were pooled into panels and the pool plates were sealed and stored at 4°C. Pooled DNA fragments generated from the amplification of microsatellite markers were separated by capillary electrophoresis using an ABI 3700 automated sequencer (PE Applied Biosystems, Inc.). Products were sized using the programme ABI GENESCAN (version 3.0) (PE Biosystems) and genotypes assigned semi-automatically using the GENOTYPER software (version 2.0).

Linkage Analysis Chromosome 3

In a genome wide screen with 384 markers (average marker distance 9.2 cm) 530 affected sib-pairs were analysed from 407 families. In addition marker genotypes for at least one parent or unaffected sibs were available in 110 families.

The genotype data for chromosome 3 include 23 markers (see Fig 1) with an average distance of 9.8 cM. Linkage analysis was performed in Genehunter-Plus version 1.3 (Kruglyak et al, Am. J. Hum. Genet. 58, 1347-1363 (1996) and Kruglyak and Lander. Journal of Computational Biology 5:1-7 (1998)) using the 'all' scoring function, followed by calculation of LOD-scores in KAC as described by Kong and Cox Am. J. Hum. Genet. 61, 1179-1188 (1997). A maximum LOD-score of 3.45 was obtained at 9 cM, with the position given as the distance from the most telomeric marker D3S1297 (Fig 1). In table 1, LOD-scores for the three markers overlapping the peak are displayed.

Position	LOD	Marker
0.0	2.51	D3S1297
9.0	3.45	-
14.1	3.21	D3S1304
27.8	0.45	D3S1263

To assess the statistical significance of the obtained LOD-score we simulated marker genotype data under a model with no genetic effect, preserving the family structures and missing data patterns of the families in the study, using the observed marker allele frequencies. In 3600 replicates no LOD-scores as large as the one observed for the real data

was observed for chromosome 3, indicating a p-value less than 0.001. Consideration of the LOD-scores obtained on all autosomal chromosomes in each replicate resulted in an empirical genome-wide p-value of 0.01.

Genetic marker distances were obtained from the Marshfield map and translated to recombination fractions using the Kosambi mapping function. The marker allele frequencies were estimated from all genotyped individuals in the study.

Prior to the linkage analysis the genotype data went through quality control. This includes identification of half-sibs or unrelated individuals among the putative sib pairs using RELPAIR (Boehnke *et al.*, Am. J. Hum. Genet. 61:423-429. 1997), detection of Mendelian inconsistencies with PEDCHECK (O'Connell *et al.*, Am. J. Hum. Genet. 63:259-266 1998) and retyping of inconsistent marker-family combinations. Detected half-sib pairs were excluded from the linkage analysis described here.

Associated gene identification

The highest linkage was found between makers D3S1297 and D3S1304, within this region lies the OXTR gene. Further SNP mapping identified this gene as being associated with the CAD.

Example 2

Identification of polymorphisms in OXTR, a coronary artery disease (CAD) susceptibility gene on chromosome 3p

Fragments of the OXTR gene were PCR amplified from genomic DNA of 24 unrelated individuals. The PCR amplified products were then sequenced and SNPs identified by Dye-primer sequencing as described in the ABI protocol P/N 402114 using ABI 3700 automated sequencers. Genotyping of informative SNPs was performed by real-time PCR using the TAQMAN™ technology from PE Biosystems.

Association analysis of OXTR CAD candidate genes in TDT samples

Genotyping results for 30 SNPs in OXTR candidate gene were analysed for association using four standard statistical methods: ASPEX, TRANSMIT, PDT and TDT-AE.

Pedigree Structure

Individuals: 1788 (863 founders, 925 non-founders)

Families: 430

Results

Two intronic SNPs in OXTR showed evidence of association, as shown in the table below.

SNP	p-value for association by different testing methods			
	TRANSMIT	ASPEX	TDT-AE	PDT
OXTR:0147B1	ns	0.023	0.0146	0.018
OXTR:0147C1	0.09	0.011	0.0167	0.002

For OXTR:0147C1 the results were either significant or approaching significance p=0.002 to p=0.09. In addition, the neighbouring SNP OXTR:0147B1 was also significant in several analyses (p=0.015 TDT-AE). The direction of the association for OXTR:0147C1 suggests a causative affect of the rare allele. However, the direction of the association for OXTR:0147B1 suggests a protective affect of the rare allele. Haplotype analysis was also carried out and one haplotype block in OXTR showed significant association (on common haplotypes; freq. >0.05), which like the analysis of OXTR:0147C1 alone is suggestive of a causative affect.

Linkage Disequilibrium/Haplotype Analysis

Five haplotype blocks were identified across the chromosome 3 region using the GOLD analysis. Two of these blocks contained the OXTR gene:

1. OXTR:0147 (T1, Z1, A2)
2. OXTR:0147 (B1, C1, D1, E1, D2, H1, M1)

TRANSMIT was run on the above haplotypes blocks (haplotype frequencies <0.05). There was one haplotype block with an overall significance p<0.05.

Gene	SNPs	Haplotype	Frequency	Observed	Expected	Robust		Bootstrap	
						X^2	p-value	X^2	p-value
OXTR	B1, C1,D1, E1, D2,H1, M1	Haplotypes >0.05		-	-	9.314 (3df)	0.025	8.8323 (3df)	0.034
OXTR	B1, C1,D1, E1, D2,H1, M1	GCTATCT	0.078	88	73	8.0527	0.0045	7.6424	0.0057

Conclusions

Significant associations have been detected in the OXTR gene.

The key SNPs and their frequencies are shown in Table 3. Sequence flanking the polymorphisms is shown in Tables 4 and 5.

The polymorphism and 30 adjacent nucleotides are provided as unique sequence identifiers (SEQ ID Nos: 3-9), which can be used to unambiguously identify the location of the polymorphism in whatever OXTR genomic sequence is used. OXTR:O147B1 is found in SEQ ID NO: 3; OXTR:O147C1 is found in SEQ ID NO: 4; OXTR:O147D1 is found in SEQ ID NO: 5; OXTR:O147E1 is found in SEQ ID NO: 6; OXTR:O147D2 is found in SEQ ID NO: 7; OXTR:O147H1 is found in SEQ ID NO: 8; and, OXTR:O147M1 is found in SEQ ID NO: 9.

Table 3

Internal identifier	Position relative to start of exon 1 (nt#1 in NM000916)	Public name	*Allele change	Allele frequency
OXTR:O147B1	15743	Rs237885	C/A	C:54% A:46%
OXTR:0147C1	10446	Rs2268493	A/G	A:70% G:30%
OXTR:0147D1	3147	Rs2268496	T/A	T:79% A:21%
OXTR:0147E1	2792	Rs237898	T/A	T:61% A:39%
OXTR:0147D2	2103	Rs237902	C/T	C:64% T:36%
OXTR:0147H1	2065	-	G/A	G:88% A:12%
OXTR:0147M1	-5043	Rs237922	T/A	T:68% A:32%

Table 4

Internal identifier	5'flanking sequence	SEQ ID NO:
OXTR:O147B1	GAGTGGCACCCCCTTCCGGTGCCTACCTAA	10
OXTR:0147C1	TTGAGATCAAGAACGGTGGACAGTTACTTT	11
OXTR:0147D1	CACCTTCAGCCTTGTCCTCAGCAGTCCTCC	12
OXTR:0147E1	TTTAAGTTCATGTTAAGATGAACTTCCACT	13
OXTR:0147D2	CGGCCTTATCAGCTTCAAGATCTGGCAGAA	14
OXTR:0147H1	GTCTACATCGTGCCGGTCATCGTGCTCGCT	15
OXTR:0147M1	ACCTGGGGAAACCAAGTCTCAGAGAAGTTC	16

Table 5

Internal identifier	3'flanking sequence	SEQ ID NO:
OXTR:O147B1	CCACAAGATGTCTGCATCGTGGTGTTTCTC	17
OXTR:0147C1	TTCATTTCTTCTTTCCTATCTATACGATTT	18
OXTR:0147D1	ACCTGGAAGGCACATTCCCCACCTACAGAA	19
OXTR:0147E1	TAAGTTCAAGAAATCCAGCTGAAGCCAAGA	20
OXTR:0147D2	TTGCGGCTCAAGACCGCTGCANCGGCGGCG	21
OXTR:0147H1	CCTGCTACGGCCTTATCAGCTTCAAGATCT	22
OXTR:0147M1	GTACCTTAGCCACGCTGACAAAACGTGGTA	23